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(71) Applicant *(for all designated States except US)*: WILLIAM HARVEY RESEARCH LIMITED [GB/GB]; Charterhouse Square, London EC1M 6BQ (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: THIEMERMANN, Christoph [DE/GB]; 42 Limehouse Cut, 46 Morris Road, London E14 6NT (GB). WANG, Jun [-/-]; 79-4F, Ting-Chou Road, Sec 3, Lane 24, LA5, Taipai (TW).

(74) Agent: HOWARD, Paul, Nicholas; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

(54) Title: USE OF ADENOSINE TRI- OR TETRA-PHOSPHATES AND THEIR ANALOGUES FOR THE TREATMENT OF CEREBRAL INFARCTION

(57) Abstract

The present invention relates to compounds for use in the treatment or prophylaxis of infarction associated with reperfusion injury, particularly cerebral infarction associated with reperfusion injury.

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USE OF ADENOSINE TRI- OR TETRA-PHOSPHATES AND THEIR ANALOGUES FOR THE TREATMENT OF CEREBRAL INFARCTION

The present invention relates to compounds for use in the treatment or prophylaxis of infarction associated with reperfusion injury, particularly cerebral infarction associated with reperfusion injury.

Infarction is most commonly due to the blockage of a nutritive blood vessel to the tissue or organ by a blood clot or thrombosis. The subsequent cessation of blood flow (ischaemia) to the tissue or organ results in the death of some of the tissue or organ.

Reperfusion by thrombolytic therapy, percutaneous transluminal angioplasty or bypass surgery, has emerged as the fundamental strategy in the management of acute ischaemic syndromes of the heart and brain. Without question, early reperfusion is an absolute prerequisite for the survival of, for example, the ischaemic myocardium. However, there is now a substantial amount of evidence that reperfusion leads to an additional injury (Forman *et al.*, Circulation, **81**, 69-78, (1990); Hearse & Bolli, Trends Cardiovasc. Med., 1, 233-240, (1991); Jeroudi *et al.*, Am. J. Cardiol., 73, 2B-7B, (1994); Hansen, Eur. Heart. J., **16**, 734-740 (1995) so that reperfusion itself can lethally damage cells. The consequences of reperfusion (leading to reperfusion injury) have been primarily investigated in the heart. It is now generally accepted that reperfusion itself triggers sudden metabolic, electrophysiologic, morphologic and functional changes which are detrimental to the myocardium. To convincingly demonstrate that a drug interferes with reperfusion-injury, injection of this drug prior to the onset of reperfusion (rather than before the onset of ischaemia) should result in a significant reduction in infarct size (Hearse & Bolli, Trends Cardiocasc. Med., **1**, 233-240, (1991)). The detrimental consequences of reperfusion, for example in the heart, include (i)

reperfusion-induced arrhythmias, (ii) myocardial stunning (iii) lethal reperfusion injury and (iv) accelerated necrosis. Although the mechanisms leading to reperfusion injury are not entirely clear, there is now a substantial amount of evidence indicating that the generation upon reperfusion of oxygen-derived free radicals and abnormalities of calcium-homeostasis (calcium overload of cells) importantly contribute to the above manifestations of reperfusion injury. Although there is some formation of radicals during ischaemia, there is a dramatic increase in the formation of oxygen-derived free radicals in the early reperfusion period. Similarly, alterations in calcium-homeostasis occur much more frequently during the reperfusion of the ischaemic myocardium. Oxygen-derived free radicals (superoxide anions, hydroxyl-radical, hydrogen peroxide) are generated upon reperfusion and cause increased membrane permeability. The increased membrane permeability allows easier access of calcium into the myocytes leading to mitochondrial calcium overload with subsequent damage to the mitochondrial structure and loss of the ability to produce adenosine triphosphate (ATP), which ultimately results in cell death. Thus, reperfusion injury is currently believed to be caused by a complex interaction between the generation of free radicals and the alterations in calcium homeostasis and therefore potentially amenable to a specific therapy aimed at reducing reperfusion injury.

The prior art is mainly directed to myocardial ischaemia which as a condition is distinct from cerebral ischaemia, reperfusion injury and especially cerebral reperfusion injury. The term myocardial ischaemia describes a condition that exists when the uptake of oxygen in the heart is insufficient to maintain the rate of cellular oxidation and metabolism. This leads to extremely complex situations, which have been extensively studied in recent years. Although there is no definite answer as to the factors determining cell death during ischaemia (without reperfusion), it is well accepted that a fall in ATP below

critical levels is of major importance. In the absence of mitochondrial injury (see above) cellular ATP levels are critically dependent on oxygen supply and oxygen demand and, hence, therapies which either reduce oxygen demand or increase oxygen supply have been shown to reduce ischaemic tissue injury. It has, however, previously been extremely difficult to delineate the mechanisms leading to ischaemic injury from the ones leading to "reperfusion-injury" as the assessment as to whether an ischaemic tissue will inevitably die can only be assessed by reperfusion of the ischaemic tissue.

The important question as to whether a specific drug or intervention reduces infarction by interfering with the mechanisms leading to ischaemic or reperfusion injury can be assessed by comparing the reduction in infarct size afforded by this drug when given either before ischaemia (with or without reperfusion) or before reperfusion. Drugs which reduce infarct size when given just prior to the onset of reperfusion clearly reduce infarct size by interfering with the events leading to reperfusion-injury.

In contrast, drugs which reduce infarct size when given during the ischaemic period (or even before, but not prior to reperfusion) are likely to give protection by causing a reduction in ischaemic tissue injury. This applies particularly to drugs which are rapidly metabolised, as they are unlikely to interfere with the consequences of reperfusion.

Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AP_4A) has been reported (European Patent Application EP-A2-0437929) as being useful in the treatment of heart disease, specifically in the treatment of arrythmia or for use as a vasodilator. Use of diadenosine 5′,5‴-P^1,P^4-tetraphosphate as an anti-thrombotic agent is also discussed in International Patent Application WO89/04321 and United States Patent 5,049,550. Diadenosine 5′,5‴-P^1,P^5 pentaphosphate (AP_5A) has been

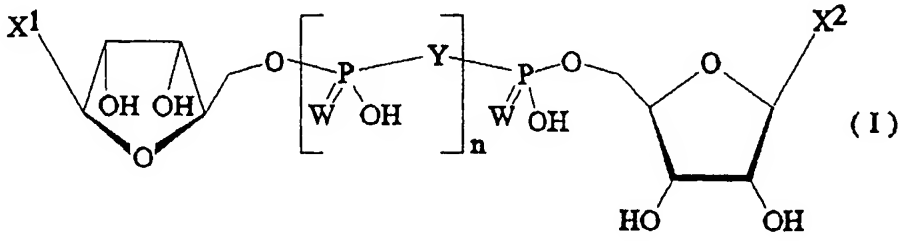
reported to be an inhibitor of adenylate kinase (G.E. Lienhard *et al.*, J. Biol. Chem., **248**, 1121 (1973); S.M. Humphrey *et al.*, Journal of Surgical Research, **43**, 1987)).

Use of diadenosine 5′,5′′′-P^1,P^4-tetraphosphate for curing ischaemic myocardial disease is disclosed in European Patent Application EP-A1-0689838. No reference to cerebral ischaemia or reperfusion injury or the reduction of cerebral infarction is made in the application.

Some analogues of 5′,5′′′-P^1,P^4 tetraphosphate have been disclosed in the prior art (Blackburn *et al.*, NAR **15**, 6994-7025, (1987)) as well as a number of analogues of 5′,5′′′-P^1,P^3-triphosphate (AP_3A) (Blackburn *et al.*, Tetrahedron letters, **31**, 5637-5640, (1990) and Guranowski *et al.*, Nucleosides and Nucleotides, **14**, 731-734, (1995)). However, there is no indication that the analogues may be useful in the treatment or prophylaxis of cerebral infarction associated with ischaemia and/or reperfusion injury.

There remains a need for improved therapeutic compounds for use in the treatment or prophylaxis of infarction especially cerebral infarction associated with reperfusion injury.

According to the present invention, there is provided use of a compound of formula (I):-



(I)

wherein X^1 and X^2 may be the same or different and each is a substituted, unsubstituted or modified purine base,

each group represented by Y may be the same or different and each is selected from the group comprising -O- and $-CZ^1Z^2-$

wherein Z^1 and Z^2 may be the same or different and each is selected from the group comprising hydrogen, halogen and alkyl groups,

each atom represented by W may be the same or different and each is selected from the group comprising oxygen and sulfur, and

n is 2 or 3,

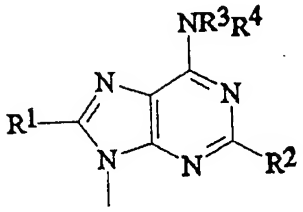
or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment or prophylaxis of cerebral infarction associated with reperfusion injury.

Infarction associated reperfusion injury is defined as the tissue necrosis or damage caused on reperfusion of an ischaemic tissue and does not include ischaemic tissue damage, namely that caused by the cessation of blood flow to the tissue.

X^1 and X^2 may be the same or different. Preferably X^1 and X^2 are the same.

X^1 and X^2 may comprise a substituted, unsubstituted or modified purine base or derivative thereof. Preferably, X^1 and X^2 comprise adenine or a derivative thereof, or guanine or a derivative thereof. Preferably, X^1 and X^2 comprise adenine or guanine, more preferably adenine.

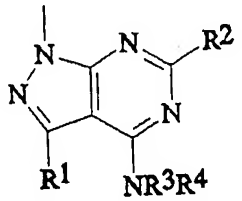
Adenine and derivatives thereof may comprise radicals of formula (II):-



(II)

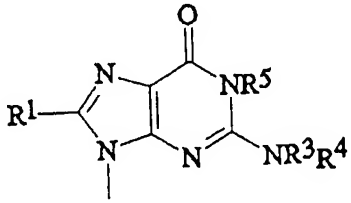
wherein R^1 and R^2 may be the same or different and are selected from the group comprising hydrogen, halogen, and alkyl, aryl, alkoxy, aryloxy, alkythio and arylthio groups, and R^3 and R^4 are the same or different and are selected from the group comprising hydrogen and alkyl, aryl, alkanoyl and aroyl groups.

Adenine and derivatives thereof may also comprise isomers of the radicals of formula (II), for example radicals of the formula (IIa):-



(IIa)

Guanine and derivatives thereof comprise compounds of the formula (III):-

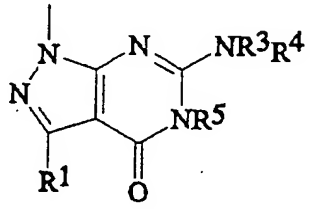


(III)

wherein R^1, R^3 and R^4 are as defined above, and R^5 is selected

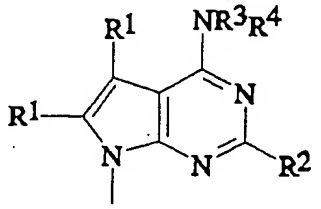
from the groups comprising hydrogen and alkyl, aryl, alkanoyl and aroyl groups.

Guanine and derivatives thereof may also comprise isomers of the radicals of formula (III), for example radicals of the formula (IIIa):-



(IIIa)

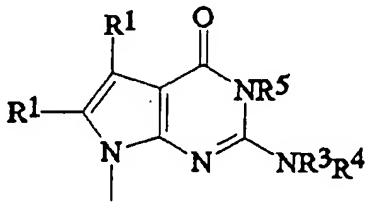
Modified purines include deazapurines. In particular, modified purines include deazaadenine and derivatives thereof which comprise radicals of formula (IV):-



(IV)

wherein R^2, R^3 and R^4 are as defined above, and the R^1 groups are independently selected from the definition of R^1 above.

Modified purines also include deazaguanine and derivatives thereof which comprise radicals of formula (V):-



(V)

wherein R^2, R^3 and R^4 are as defined above, and the R^1 groups are independently selected from the definition of R^1 above.

Other modified purines which will be useful in the present invention will be apparent to those skilled in the art.

Reference in the present specification to alkoxy and aryloxy groups means alkyl-O- and aryl-O- groups, and their haloalkyl-O- and haloaryl-O- groups, respectively. Reference to alkanoyl and aroyl groups means alkyl-CO- and aryl-CO-, respectively. Reference in the present specification to an alkyl group means a branched or unbranched, cyclic or acyclic, saturated or unsaturated (e.g. alkenyl or alkynyl) hydrocarbyl radical. Where cyclic, the alkyl group is preferably C_3 to C_{12}, more preferably C_5 to C_{10}, more preferably C_5 to C_7. Where acyclic, the alkyl group is preferably C_1 to C_{10}, more preferably C_1 to C_6, more preferably methyl, ethyl, propyl or a halo-derivative thereof.

Reference in the present specification to an aryl group means an aromatic group, such as phenyl or naphthyl, or a heteroaromatic group containing one or more, preferably one, heteratom, such as pyridyl, pyrrolyl, furanyl and thiophenyl. Preferably, the aryl group comprises phenyl.

The alkyl and aryl groups may be substituted or unsubstituted, preferably unsubstituted. Where substituted, there will generally be 1 to 3 substituents present, preferably 1 substituent. Substituents may include halogen atoms; oxygen containing groups such as oxo, hydroxy, carboxy, carboxyalkyl, alkoxy, alkoyloxy; nitrogen containing groups such as amino, alkylamino, dialkylamino, cyano, azide and nitro; sulfur containing groups such as thiol, alkythiol, sulphonyl and sulphoxide; heterocyclic groups containing one or more, preferably one, heteratom, such as thiophenyl, furanyl, pyrrolyl, imidazolyl, pyrazolyl, thiazolyl, isothiazolyl, oxazolyl, pyrrolidinyl, pyrrolinyl, imidazolidinyl, imidazolinyl, pyrazolidinyl, tetrahydrofuranyl, tetrahydrothiophenyl, pyranyl, pyronyl, pyridyl, pyrazinyl, pyridazinyl, piperidyl, piperazinyl,

morpholinyl, thionaphthyl, benzofuranyl, isobenzofuryl, indolyl, oxyindolyl, isoindolyl, indazolyl, indolinyl, 7-azaindolyl, isoindazolyl, benzopyranyl, coumarinyl, isocoumarinyl, quinolyl, isoquinolyl, naphthyridinyl, cinnolinyl, quinazolinyl, pyridopyridyl, benzoxazinyl, quinoxadinyl, chromenyl, chromanyl, isochromanyl and carbolinyl; and aryl groups such as phenyl and substituted phenyl. Alkyl includes substituted and unsubstituted benzyl.

Reference in the present specification to halogen means a fluorine, chlorine, bromine or iodine radical, preferably fluorine or chlorine radical.

Each group represented by Y may be the same or different and each is selected from the group comprising -O- (oxygen) and $-CZ^1Z^2-$ (substituted or unsubstituted methylene radicals). Preferably Y are not the same and preferably at least one is -O- and the other or others is/are CZ^1Z^2.

Each Y may comprise $-CZ^1Z^2-$ wherein Z^1 and Z^2 may be the same or different and each is selected from the group comprising hydrogen, halogen and alkyl groups. Preferably $-CZ^1Z^2-$ is CCl_2, CHCl, CF_2, CHF or CH_2.

Each atom represented by W may be the same or different and each is selected from the group comprising oxygen and sulfur. Preferably, W are the same and each are oxygen.

Preferably, the compound for the use of the present invention is diadenosine 5'5'''-P^1,P^3-substituted triphosphate or diadenosine 5'5'''-P^1,P^4-substituted tetraphosphate, and more preferably $APCCl_2PCCl_2PA$, $APCF_2PCF_2PA$, APCHFPCHFPA, APCHClPCHClPA, $APCH_2PCH_2PA$, $APCCl_2PPCCl_2PA$, $APCF_2PPCF_2PA$, APCHFPPCHFPA, APCHClPPCHClPA or $APCH_2PPCH_2PA$.

Reference to cerebral infarction associated with reperfusion

injury means tissue necrosis or damage to cerebral tissue arising from the reperfusion of ischaemic cerebral tissue with blood. "Reperfusion injury", as indicated previously, is thought to be due to the invasion of injured tissue with neutrophils (white blood cells), which then become activated and cause the release of oxygen radicals and enzymes. A particular feature of the present invention is the prophylactic protection of cerebral tissue afforded by the compounds of the invention against infarction associated with reperfusion injury. This feature makes the compounds of the invention particularly useful in the prophylaxis of infarction associated with reperfusion injury in conditions associated with interruption on the blood supply to cerebral tissue and subsequent reperfusion (for example, thrombosis, hypoperfusion due to surgery, trauma etc.). The compounds of the present invention are also particularly useful in the prophylaxis of conditions of cerebral reperfusion such as stroke.

The medicaments employed in the present invention can be administered by oral or parenteral route, including intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, airway (aerosol), rectal and topical administration.

For oral administration, the compounds of the invention will generally be provided in the form of tablets or capsules or as an aqueous solution or suspension.

Tablets for oral use may include the active ingredients mixed with pharmaceutically acceptable excipients such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavouring agents, colouring agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatin, while the lubricating agent, if

present, will generally be magnesium stearate, stearic acid or talc. If desired, the tablets may be coated with a material such as glyceryl monostearate or glyceryl distearate, to delay absorption in the gastrointestinal tract.

Capsules for oral use include hard gelatin capsules in which the active ingredient is mixed with a solid diluent, and soft gelatin capsules wherein the active ingredient is mixed with water or an oil such as peanut oil, liquid paraffin or olive oil.

For intramuscular, intraperitoneal, subcutaneous and intravenous use, the compounds of the invention will generally be provided in sterile aqueous solutions or suspensions, buffered to an appropriate pH and isotonicity. Suitable aqueous vehicles include Ringer's solution and isotonic sodium chloride. Aqueous suspensions according to the invention may include suspending agents such as cellulose derivatives, sodium alginate, polyvinyl-pyrrolidone and gum tragacanth, and a wetting agent such as lecithin. Suitable preservatives for aqueous suspensions include ethyl and n-propyl p-hydroxybenzoate.

The compounds of the invention may also be presented as liposome formulations.

The compounds of the present invention may be presented alone or in combination with thrombolytic agents such as t-PA or streptokinase, or with agents such as prostacyclin, nitric oxide donors, organic nitrates, calcium antagonists, inhibitors of the activity of poly (ADP-ribose) synthetase (PARS) or nitric oxide synthase inhibitors.

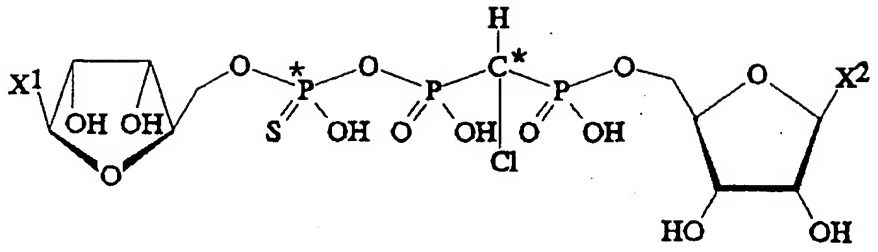
The invention further provides use of a compound of formula (I) or an analogue thereof or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment or prophylaxis of inflammation associated with

reperfusion injury of cerebral tissue.

According to a further aspect of the present invention there is a method of treatment or prophylaxis of cerebral tissue infarction or inflammation associated with reperfusion injury comprising adminstration to a patient, an effective dose of a compound of formula (I) or a pharmaceutically acceptable salt thereof.

According to a further aspect of the present invention there is provided a compound of formula (I) wherein n is 2 and at least one of the atoms represented by W is S, or a pharmaceutically acceptable salt thereof. Preferably, the compound of the present invention is APSPCCl$_2$PA, APSPCF$_2$PA, APSPCHClPA or APSPCH$_2$PA.

The compounds of the present invention and the compounds used in the present invention may exist as a number of stereoisomers. For example, APSPCHClPA has stereoisomeric centres at P and C in the phosphate moiety, which are marked with an asterisk in the formula below, and therefore has 4 stereoisomers for this phosphate moiety.



The invention will now be described with reference to the following example and to the figures in which:-

Figure 1 illustrates the effect of AP_4A administered prior to onset of ischaemia on: (A) volume of cerebral infarction (expressed in mm^3); (B) area of largest infarction in one single brain slice (mm^2); (C) the number of infarcted slices per rat brain; and (D) the incidence of infarction within the whole group of animals tested.

Figure 2 illustrates the effect of AP_4A administered prior to onset of ischemia and the effect of AP_4A administered prior to onset of reperfusion on: (A) volume of cerebral infarction (expressed in mm^3); (B) area of largest infarction in one single brain slice (mm^2); (C) the number of infarcted slices per rat brain; and (D) the incidence of infarction within the whole group of animals tested.

Figure 3 illustrates the effects of adenosine administered prior to onset of reperfusion on: (A) volume of cerebral infarction (expressed in mm^3); (B) area of largest infarction in one single brain slice (mm^2); (C) the number of infarcted slices per rat brain; and (D) the incidence of infarction within the whole group of animals tested.

It will be appreciated that what follows is by way of example only and that modifications to detail may be made whilst still falling within the scope of the invention.

EXPERIMENTAL

Selection and treatment of animals

A total of 37 adult male Sprague-Dawlay rats (weight 375 ± 10g) were used. The animals were anaesthetised with chloral hydrate (400mg/kg, i.p.). After anaesthesia, a compound of the invention or its vehicle (phosphate-buffered saline) was injected into the left lateral cerebral ventricle at a volume of 10μl. Ten minutes after this i.c.v. injection, the squamosal bone overlying the right frontal and temporal cortex was removed (an area of approximately 2 x 2 mm^2). Subsequently, three injections of the compound of the invention or its vehicle (5μl into three sites) were made directly into the cortex (1 mm below the cortical surface) adjacent to the middle cerebral artery (MCA) by using a Hamilton syringe. Five minutes after intracortical injection, the left MCA and both common carotid arteries were ligated for 90 minutes according to the method described by Chan *et al.*, (Stroke **17**, 738-743 (1986)). Briefly, the bilateral common carotid arteries were identified and isolated (through a ventral midline cervical incision). The carotid arteries were subsequently occluded with non-traumatic arterial clips. Following a craniotomy of about 2 x 2 mm^2 (which was made in the right squamosal bone), the right MCA was ligated with a 10-O suture for ninety minutes. As previously reported, a ninety minute ligation of this artery induces a maximal cerebral infarction in the rat (Du *et al.*, J Cereb Blood Flow Metab **16**, 195-201 (1996)). After ninety minutes of MCA occlusion, the clip was removed to allow a subsequent twenty four hour reperfusion period. At the end of this reperfusion period, the animal was killed by an overdose of anaesthetic and then received an infusion of saline solution which was administered intracardially. Subsequently, the brain was removed, immersed in cold saline for five minutes and sliced into 2 mm sections. The brain slices were then incubated in a 2% tri-phenyl-tetrazoliun chloride (TTC) dissolved in

phosphate buffered saline (thirty minutes at 37°C) and then transferred to 5% formaldehyde solution for fixation (Chan *et al*. 1986). The volume of infarction was measured in each slice and calculated by using the image tools (version 1.27) programme provided by the University of Texas Health Science Centre.

Drug Regimen

The 37 animals were divided into the following groups:

(1) Administration of vehicle (0.1M phosphate buffered saline, PBS): Following an injection of 25μl of vehicle, three further injections of this vehicle (5μl each) were made directly into the cerebral cortex, (n=13).

(2) Administration of AP_4A prior to ischaemia: All animals subjected to the treatment group received prior to onset of ischaemia a total dose of 2.5μg of AP_4A dissolved in 25μl of 0.1M PBS; 10μl of this solution of AP_4A (containing 10μg of the drug) were injected i.c.v., while three injections (5μl each, containing 5μg of AP_4A) were subsequently made directly into the cerebral cortex.

(3) Administration of AP_4A prior to reperfusion: All animals randomised to this treatment group received immediately prior to the onset reperfusion a total dose of 2.5 μg of AP_4A dissolved in 24 μl of 0.1 M PBS: 10μl of this solution of AP_4A (containing 10μg of the drug was injected i.c.v., while three injections (5μl each, containing 5μg of AP_4A) were subsequently made directly into the cerebral cortex.

(4) Administration of adenosine: All animals randomised to this treatment received immediately prior to reperfusion a total dose of 2.5μg of adenosine dissolved in 25μl of 0.1M PBS: 10μl of this solution of adenosine (containing 10μg of the drug) were injected i.c.v., while three injections (5μl each containing 5μg of adenosine) were subsequently made directly into the cerebral cortex.

Statistical Analysis

All values in Figures in text are expressed as the mean ±

s.e. mean of n observations. The statistical analysis in Figure 1 (A), (B) and (C) was carried out by an unpaired Students-t test while the statistical analysis in Figure 1 (D) was carried out by means of Fisher-Exact test. The statistical analysis in Figure 2 (A),(B) and (C) was carried out by ANOVA followed by a Bonferoni test for multiple comparison, while the statistical analysis in Figure 2 (D) was carried out by means of a Fisher-Exact test. The statistical analysis in Figure 3 (A to C) was carried out by an unpaired Student's t-test, while the statistical analysis in Figure 3 (D) was carried out by means of a Fisher-Exact test. A p value of less than 0.05 was considered statistically significant and is indicated with an asterisk.

Synthesis of Analogues AP_4A and AP_3A

Compounds of the present invention which are not commercially available may be prepared according to conventional techniques, such as described in Blackburn *et al.*, NAR, **15**, 6991-2025, (1987); Guranowski *et al.*, Nucleosides and Nucleotides, **14**, 731-734, (1995); and Blackburn *et al.*, Tetrahedron Letters, **31**, 5637-5640, (1990) the teachings of which are incorporated herein by reference.

<u>Preparation of Adenosine 5'-P^1,P^2-dichloromethylene bisphosphate</u>

Adenosine 5'-P^1,P^2-dichloromethylenebisphosphate was prepared from adenosine (as described in Davisson, V.J. *et al.*, J. Org. Chem., **52**, 1794-1801 (1987) for adenosine 5'-α,β-difluoromethylene-bisphosphate) but using dichloromethylenebisphosphonate in place of difluoromethylenebisphosphonate. Yield 46 %. Analytical data: NMR δ_p (D_2O): 11.1 (d, J 16.7 Hz, P^1) and 8.75 (d, J 16.7 Hz, P^2). δ_H (D_2O): 8.47 (s, H^8), 7.95 (s, H^2),6.0 (d, J5, H^1), 4.70 (t, J4.3, $H^{2'}$), 4.55 (t, J4.4, $H^{3'}$), 4.43 (t, J4.4, $H^{5'}$,$H^{5''}$), and 4.38 (m, $H^{4'}$). FAB-MS(negative): m/z 562 (10%, M-H^+), 560 (22%, M-H^+),558 (22%, M-H^+), 540 (29%,

$M-Na^+$), 538 (89%, $M-Na^+$) and 536 (100%, $M-Na^+$). $C_{11}H_{12}Cl_2N_5O_9P_2Na_3$ has MW 563, 561, 559 for the three isotopes of chlorine.

Preparation of Diadenosine 5′,5′′′-(P^1,P^2-dichloromethylene-P^3-thio)-P^1,P^3-trisphosphate (APSPCCl_2PA).

Adenosine 5′-thiophosphate (180 mg, 0.308 mmol) as its bis-triethylammonium salt and tri-n-octylamine (0.141 ml, 0.323 mmol) were shaken in methanol (7 ml) until dissolution was achieved. The solution was evaporated under reduced pressure. The residue was then coevaporated with pyridine (3 x 10 ml) and further dried under vacuum over P_2O_5 for 12 h. The oily residue was then dissolved in dry dioxane (3 ml). Diphenyl phosphorochloridate (0.098 ml, 0.471 mmol) and tri-n-butylamine (0.176 ml, 0.739 mmol) were added. The mixture was stirred at rt. and the initial cloudy solution gradually became clear. After 3.5 h, the solvent was evaporated and the oily residue was washed with dry diethyl ether (3 x 10 ml) and then coevaporated with dry pyridine (2 x 10 ml). Adenosine 5′-(P^1,P^2-dichloromethylene) diphosphate (165 mg, 0.237 mmol, prepared as described in Davisson et al.) as its tris-triethylammonium salt and tri-n-butylamine (0.113 ml, 0.474 mmol) were shaken in dry methanol (5 ml) until dissolution was achieved, then the solution was evaporated under reduced pressure. The residue was coevaporated with pyridine (3 x 10 ml) and further dried in vacuo over P_2O_5 overnight. The resulting oil was dissolved in dry pyridine (3.6 ml) and this solution was added to the nucleoside activated as above. The reaction mixture was stirred overnight at rt., then evaporated under reduced pressure. The oily residue was partitioned between dichloromethane (2 x 15 ml) and water (50 ml), the aqueous layer was evaporated under reduced pressure and the residue was chromatographed on a DEAE A-25 Sephadex column with gradient elution using aqueous triethylammonium hydrogen carbonate (TEAB) solution pH 7.6 from 0.05 M to 0.5 M in 4 litres. The product, as its triethylammonium salt, was

eluted at a concentration of 0.37 M TEAB. The product-containing fractions were pooled and evaporated under reduced pressure. The residue was coevaporated with methanol (3 x 15 ml) and the compound was obtained as its triethylammonium salt. To converted this into its sodium salt, the product was dissolved in 2 ml methanol and added dropwise into a stirred solution of NaI in acetone (50 ml, 1 M). The precipitate was collected by centrifugation and washed with acetone (4 x 50 ml). Yield 97 mg (45% as trisodium salt). Spectroscopic and analytical data are as follows:

NMR δ_P (D_2O): 43.8 (d, J 34.5) and 43.5 (d, J 34.5) (P^3, two diastereoisomers), 8.5 (d, J 20.0, P^1), -1.4 (dd, J 20.0 and 34.5) and -1.5 (dd, J 20.0 and 34.5) (P^2, two diastereoisomers). δ_H (D_2O): 8.45 (s), 8.42 (s), 8.40 (s) and 8.36(s) [2H in total], 8.04 (m, 2H), 5.97 (m, 2H) and 4.56-4.25 (m, 10H). FAB-MS(positive): m/z 839 ($M+H^+$), 861 ($M+Na^+$) and 883 ($M+2Na^+-H^+$).

Preparation of Diadenosine 5′,5′′′-(P^1,P^2-methylene-P^3-thio)-P^1,P^3-trisphosphate (APSPCH_2PA).

This compound was prepared similarly to diadenosine 5′,5′′′-(P^1,P^2-dichloromethylene-P^3-thio)-P^1,P^3-trisphosphate (above). Adenosine 5′-thiophosphate (226 mg, 0.4 mmole) as its tris-triethylammonium salt and tri-n-octylamine (179 mg, 0.48 mmole) was activated with diphenyl phosphorochloridate (252 mg, 1.2 mmole) and tri-n-butylamine (0.48 ml) using the methods described for the preparation of diadenosine 5′,5′′′-(P^1,P^2-dichloromethylene-P^3-thio)-P^1,P^3-trisphosphate in the above section. This was condensed with adenosine 5′-methylenebisphosphonate (221 mg, 0.4 mmole) as its tri-n-butylammonium salt as described above. Chromatography of the crude product on Sephadex A-25 with a TEAB gradient from 0.05 M to 0.6 M gave the pure product as a white powder (75 mg, 20 %) with recovery of 61% of unreacted adenosine 5′-P^1,P^2-methylenebisphosphonate as its

-tris-triethylammonium salt (136 mg, 0.25 mmol). Analytical hplc showed this material to be a mixture of 2 diastereoisomers, as also evident in the ^{31}P NMR signals. This was converted into the trisodium salt as described above (58 mg, 18 % yield). Analytical data are: δ_p (D_2O): 43.0 (d, J 31.4) and 43.3 (d, J 31.4) (P^3, two diastereoisomers), 17.9 (d J 8.4) and 18.0 (d, J 7.8) (P^1, two diastereoisomers), 7.6 (dd, J 31.2 and 7.9, P^2). δ_H (D_2O): 8.50 (s), 8.43 (s), 8.35 (s) and 8.31 (s) [2H in total], 8.02 (m, 2H), 5.91-6.01 (m, 2H), 4.05-4.74 (m, 10H), 3.28 (m, 2H, PCH_2P). FAB-MS (positive): m/z 771 ($M+H^+$), 793 ($M+Na^+$), 815 ($M+2Na^+-H^+$), 837 ($M+3Na^+-2H^+$).

Results

There is now good evidence that ligation of the MCA in the anaesthetised rat (using a protocol identical to the one used in this study) causes cortical infarctions (Chan *et al.*, 1986; Du *et al.*, 1996). Here we demonstrate that injection of the vehicle (0.1M PBS) does not cause a significant alteration of cerebral infarct size caused by MCA ligation and reperfusion. This finding confirms previous studies by Wang *et al.*, (J. Neurosci **17**, 4341-4348 (1997)). However, pre-treatment of rats with AP_4A caused a significant reduction in the volume of cortical infarction as measured by TTC staining (Figure 1 (A)). In all of the PBS-control animals studied (n=13), MCA ligation and reperfusion resulted in a substantial infarction of the cortex. In contrast, only four out of nine rats which had received AP_4A as pre-treatment showed a mild degree of infarction after MCA ligation and reperfusion. The incidence and the volume of the infarction was significantly reduced by AP_4A treatment (Figure 1 (A) and (D)). Furthermore, the number of infarcted slices in the brain was significantly reduced by AP_4A from 5.3 ± 0.5 slices per rat (in PBS treated control rats) to 2.3 ± 0.8 slices per rat (in AP_4A treated rats, $p<0.05$) (Figure 1 (C)). The area of the largest infarction (observed in any slice obtained in

-one individual brain) was similarly reduced from 15.2 ± 1.22 mm^2 (in PBS control rats) to 7.1 ± 2.5 mm^2 in rats treated with AP_4A ($P<0.05$) (Figure 1 (B)).

In addition, the results demonstrate that administration of AP_4A immediately prior to reperfusion also causes a significant reduction in the volume of cerebral infarction (Figure 2A and Table 1). Moreover, administration of AP_4A immediately prior to reperfusion also caused a significant reduction of the area of the largest infarction in one slice (Figure 2B and Table 1), significantly reduced the number of infarcted brain slices which were observed in one individual brain (Figure 2C and Table 1) and also significantly reduced the overall incidence of infarction in the number of animals studied (Figure 2D). Please note that the reduction in infarct size afforded by AP_4A when given either prior to ischaemia or prior to reperfusion is identical (see Figure 2 and Table 1). These findings demonstrate that the observed reduction in infarct size afforded by AP_4A is due to a prevention of "reperfusion-injury" rather than ischaemic injury by this diadenosine nucleotide.

As AP_4A is metabolised to adenosine, it has been investigated whether the protective effects of AP_4A observed in the study are mediated by adenosine. It is demonstrated, however, that administration of adenosine immediately prior to reperfusion does not cause a significant reduction in infarct size (Figure 3 and Table 2). This finding conclusively demonstrates that (i) the metabolism of AP_4A to adenosine is not a necessary requirement for the reduction in cerebral infarct size afforded by this agent and (ii) adenosine does not reduce cerebral infarct size caused by ischaemia and reperfusion of the rat brain.

TABLE 1

	PBS	AP_4A Pre-ischemia	AP_4A Pre-reperfusion
[a]Incidence of infarction	13/0	4/5	4/4
[b]Volume of infarction/rat (mm^3)	123.0±14.0	*56.0±24.6	*51.4±22.3
# of infarcted slices/rat	5.3±0.5	*2.3±1.0	*3.0±1.2
Area of the largest infarction in one slice/rat (mm^2)	15.2±1.2	*7.1±3.1	*7.1±2.7
Body Weight	392.9±11.7	337.3±12.1	346.8±12.5
# of animals studied largest	13	9	8

Area of infarction was calculated after TTC staining.

[a]Number of animals with infarction/number of animals without infarction.

[b]Volume of infarction=2 mm (thinkness of the slice) x (sum of the infarction area in all brain slices mm^2)

*P<0.05 one way ANOVA=Bonferroni's test

Effect of AP_4A administered prior to onset of ischemia or reperfusion.

TABLE 2

	PBS	Adenosine
[a]Incidence of infarction	13/0	6/1
[b]Volume of infarction/rat (mm^3)	123.0±14.0	115.85±26.8
# of infarcted slices/rat	5.3±0.5	5.2±1.1
Area of the largest infarction in one slice/rat (mm^2)	15.2±1.2	12.9±2.7
Body weight	392.9±11.7	363.8±4.2
# of animals studied	13	7

Area of infarction was calculated after TTC staining.

[a]Number of animals with infarction/number of animals without infarction.

[b]Volume of infarction=2 mm (thinkness of the slice) x (sum of the infarction area in all brain slices mm^2)

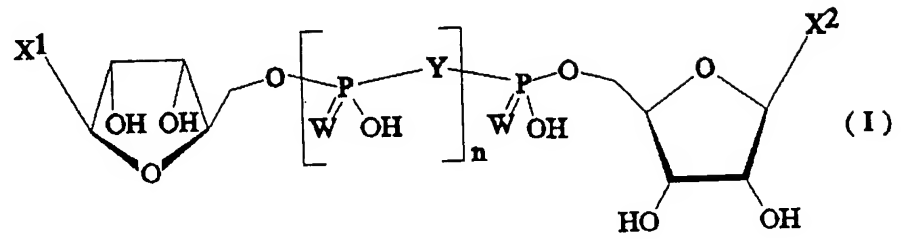
Effect of adenosine administered prior to onset of reperfusion.

Conclusion

The data clearly demonstrate that administration of AP_4A (either prior to ischaemia or prior to reperfusion) reduces the extent of infarction in rat brains subjected to ischaemia reperfusion injury. The finding that the degree of infarct size reduction afforded by AP_4A given prior to the onset of reperfusion is identical to the one of AP_4A given prior to the onset of cerebral ischaemia demonstrates that AP_4A reduces the extent of reperfusion injury (rather than ischaemic injury) in this model of cerebral infarction.

- **CLAIMS:**

1. Use of a compound of formula (I)



(I)

wherein X^1 and X^2 may be the same or different and each is a substituted, unsubstituted or modified purine base,

each group represented by Y may be the same or different and each is selected from the group comprising -O- and $-CZ^1Z^2-$

wherein Z^1 and Z^2 may be the same or different and each is selected from the group comprising hydrogen, halogen and alkyl groups,

each atom represented by W may be the same or different and each is selected from the group comprising oxygen and sulfur, and

n is 2 or 3,

or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment or prophylaxis of cerebral infarction associated with reperfusion injury.

2. Use of a compound according to claim 1 where X^1 and X^2 may be the same or different and is each adenine or a derivative thereof or guanine or a derivative thereof.

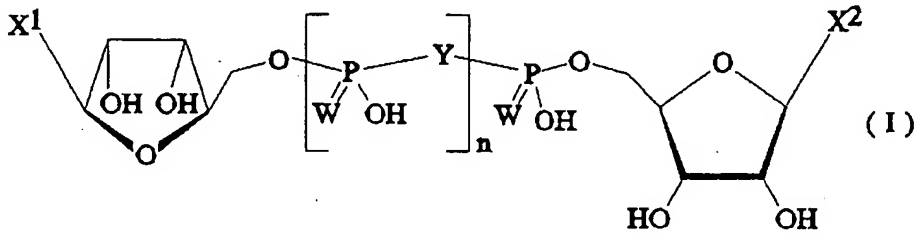
3. Use of a compound according to claim 1 where X^1 and X^2 may be the same or different and is each a modified adenine or a modified guanine.

4. Use of a compound according to any preceding claim, wherein X^1 and X^2 are the same.

5. Use of a compound according to claim 4 wherein the compound is diadenosine 5′, 5′′′-P^1,P^3-triphosphate or diadenosine 5′, 5′′′-P^1,P^4-tetraphosphate.

6. Use of the compound according to claim 4 wherein the compound is $APCCl_2PCCl_2PA$, $APCF_2PCF_2PA$, APCHFPCHFPA, APCHClPCHClPA, $APCH_2PCH_2PA$, $APCCl_2PPCCl_2PA$, $APCF_2PPCF_2PA$, APCHFPPCHFPA, APCHClPPCHClPA or $APCH_2PPCH_2PA$.

7. A compound of formula (I)



(I)

wherein X^1 and X^2 may be the same or different and each is a substituted, unsubstituted or modified purine base,

each group represented by Y may be the same or different and each is selected from the group comprising -O- and $-CZ^1Z^2-$

wherein Z^1 and Z^2 may be the same or different and each is selected from the group comprising hydrogen, halogen and alkyl groups,

each atom represented by W may be the same or different and each is selected from the group comprising oxygen and sulfur provided at least one atom is sulfur, and is 2 or 3,

or a pharmaceutically acceptable salt thereof.

8. A compound according to claim 7 for use in a method of treatment or prophylaxis of cerebral infarction associated with reperfusion injury.

9. A method of treatment or prophylaxis of cerebral infarction associated with reperfusion injury comprising administration to a patient or organ, an effective dose of a compound of formula (I) or pharmaceutically acceptable salt thereof.

FIG. 1(A)

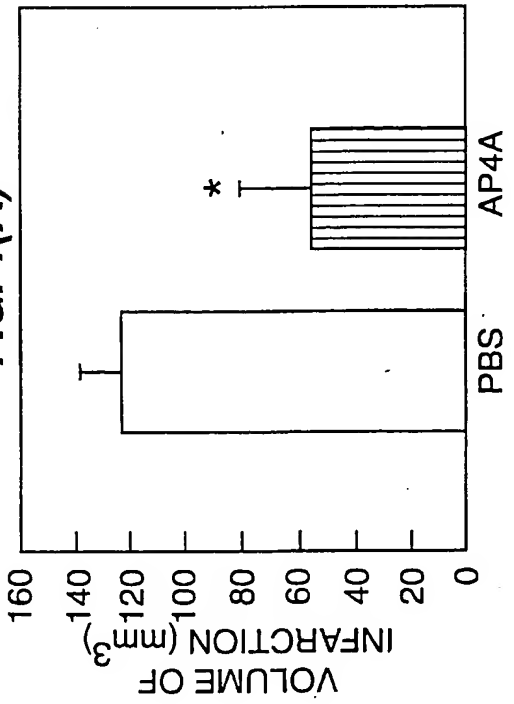


FIG. 1(B)

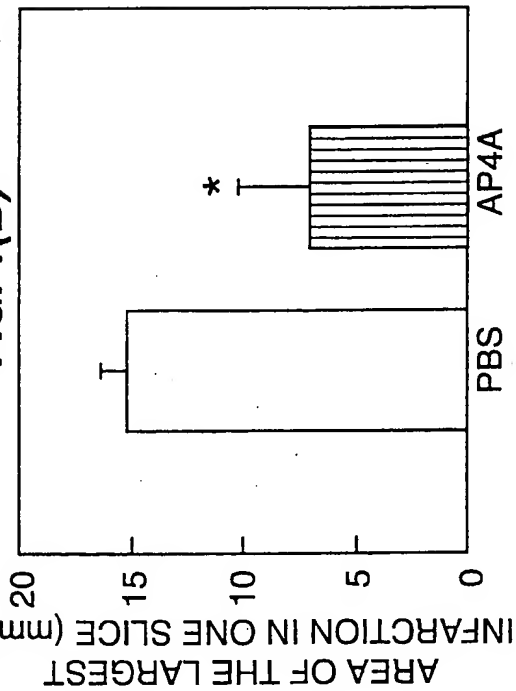


FIG. 1(C)

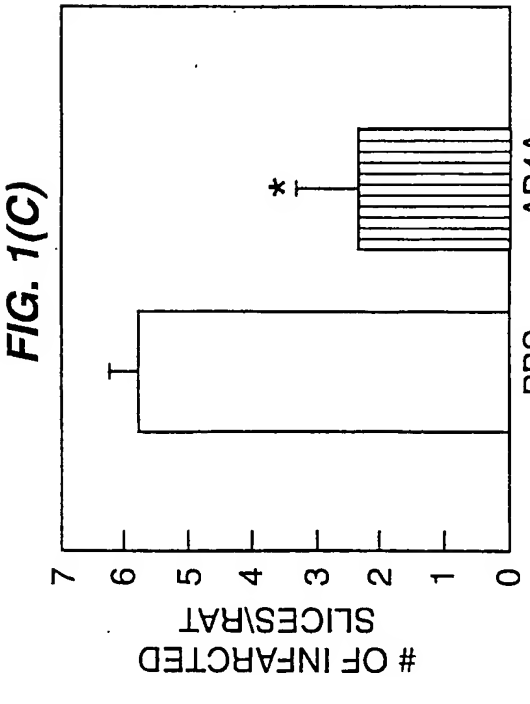
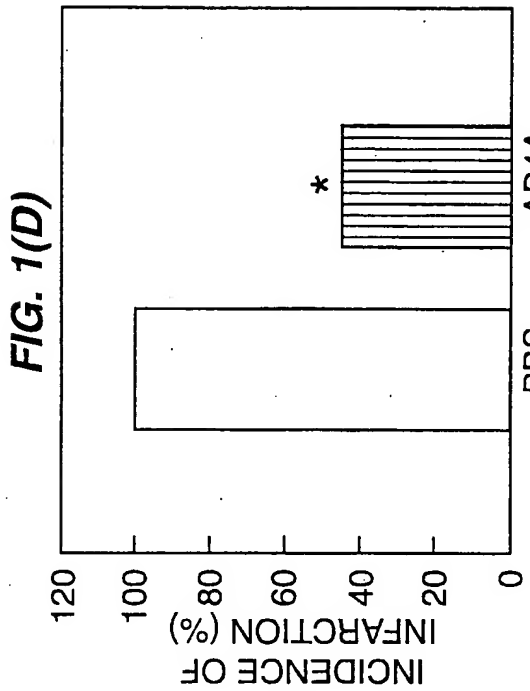
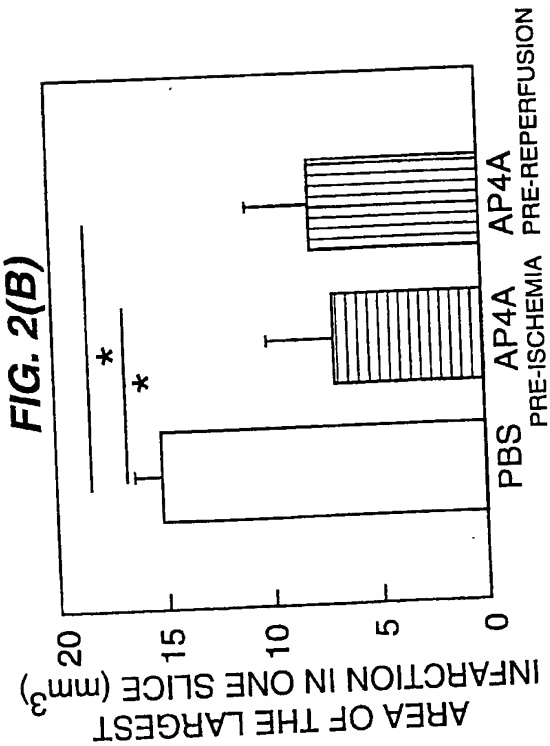
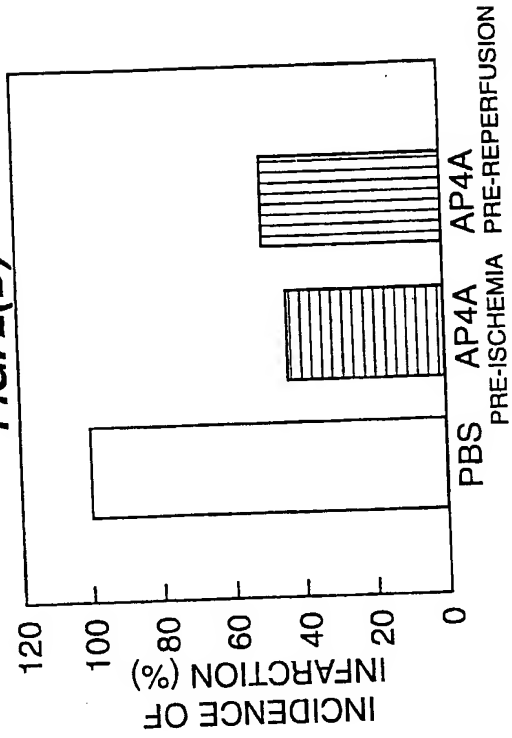
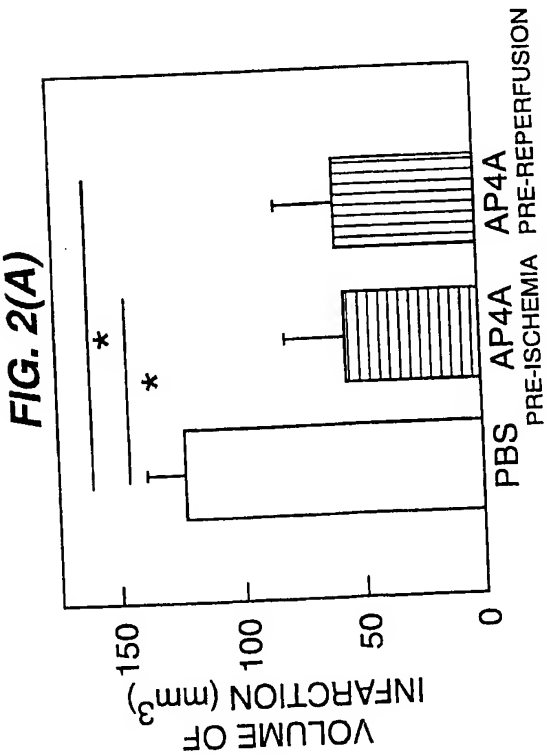


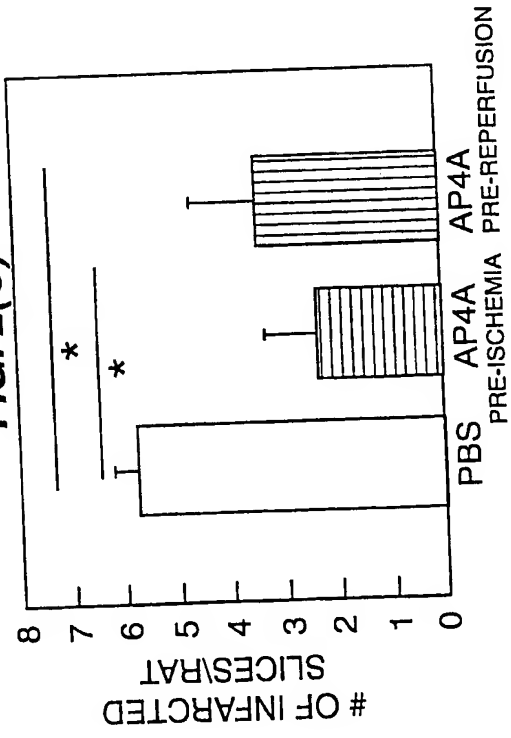
FIG. 1(D)

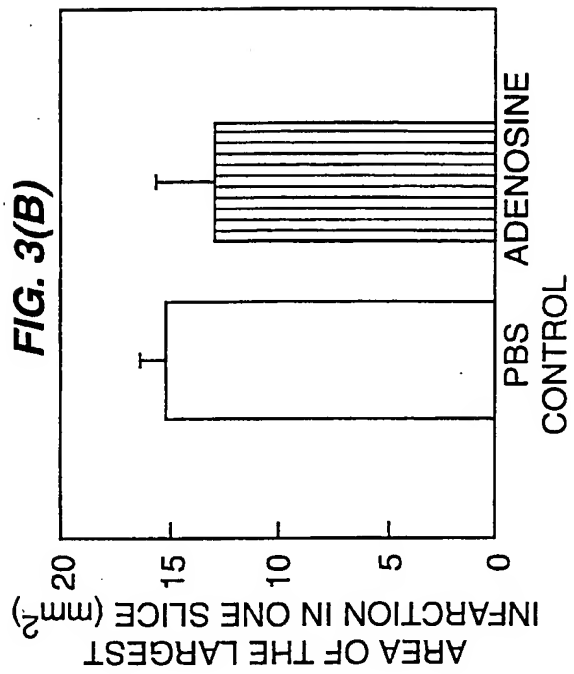


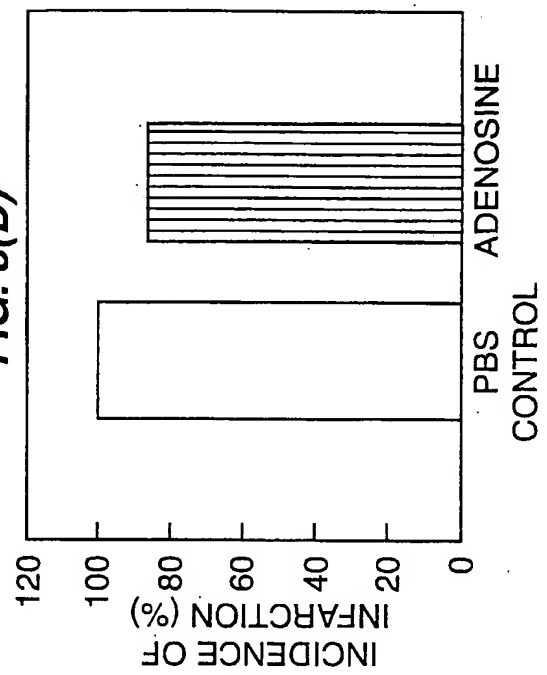


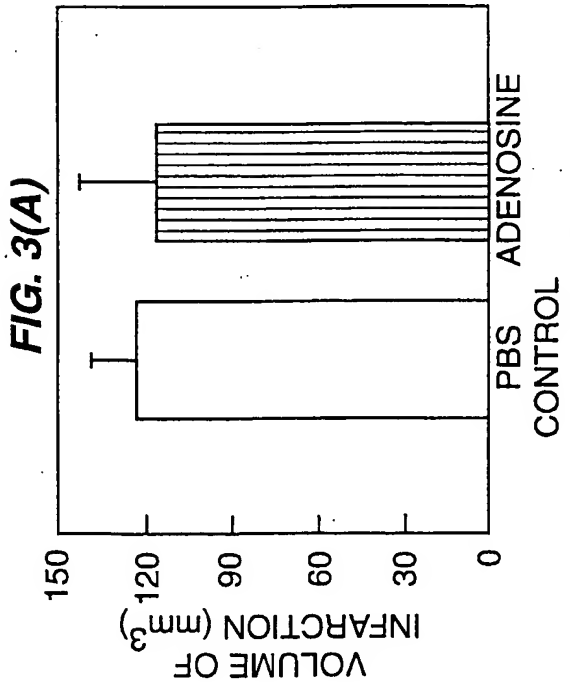


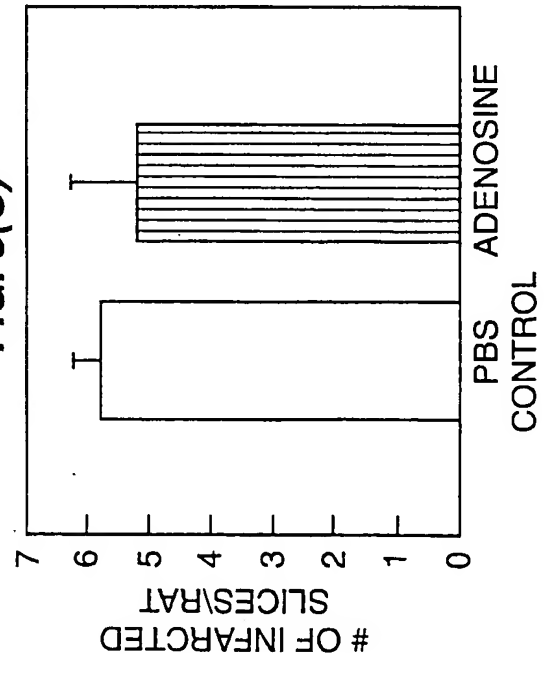












INTERNATIONAL SEARCH REPORT

Intl. Jonal Application No
PCT/GB 98/02101

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	---	1-6,9
X	WO 96 40059 A (STUTTS MONROE J III ;UNIV NORTH CAROLINA (US); GEARY CARA A (US);) 19 December 1996 see claims 1-6	7,8
Y	---	1-6,9
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 September 1998	08/10/1998

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Int Jonal Application No
PCT/GB 98/02101

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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X	---	7,8
X	EP 0 437 929 A (FUJIREBIO KK ;UNITIKA LTD (JP)) 24 July 1991 cited in the application see page 4, column 6-8; examples 1-7	7,8
Y	---	1-6,9
X,P	WO 97 40840 A (KIM BYUNG K ;PRP INC (US); ZAMECNIK PAUL C (US)) 6 November 1997 see claims 1-14	7,8
Y	---	1-6,9

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INTERNATIONAL SEARCH REPORT

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